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(54) Title: COMPOSITION

(57) Abstract: The present invention relates to adjuvant compositions which are suitable to be used in vaccines. In particular, the adjuvant compositions of the present invention comprises a Yersinia adhesion protein, optionally with a carrier. Also provided by the present invention are vaccines comprising the adjuvants of the present invention and an antigen. Further provided are methods of manufacture of the adjuvants and vaccines of the present invention and their use as medicaments. Methods of treating an individual susceptible to or suffering from a disease by the administration of the vaccines of the present invention are also provided.

Composition

The present invention relates to a new adjuvant system comprising an adhesion protein from Yersinia species, fragments and peptides thereof. The new adjuvant systems are suitable for mucosal administration into humans. Also provided is a vaccine composition comprising the new adjuvant system and an antigen or an antigenic composition. The present invention also relates to a novel use of an adhesion protein from Yersinia species as an adjuvant, fragments and peptides thereof. The present invention provides said novel adjuvants, methods of their manufacture and their formulation into mucosal vaccines. The use of the mucosal adjuvants or vaccines of the present invention in the prophylaxis or therapy of disease is also provided.

Yersinia is a gram-negative bacteria which comprises several species. Among them, three species of Yersinia are generally recognised as being enteropathogens for humans and rodents: *Yersinia enterocolitica, Yersinia pestis* and *Y. pseudotuberculosis*. They are taken up with contaminated food. Yersiniae cross the intestinal epithelium via translocation through M cells, which are located in the follicle-associated epithelium (FAE) of Peyer's patches (PP).

Yersinia main adhesion protein, the outer membrane invasin (Inv) protein, plays a major role in the invasion process and therefore in the associated pathogenesis. Through its interaction with the β-1 integrin expressed at the surface of M cells, it allows the bacterium translocation into PP (Clark et al., Infect Immun, 66, 1237-43, 1998). The main adhesion protein from *Y. pseudotuberculosis* and *Yersinia enterocolitica* are encoded by a gene referred to as inv, they are structurally similar proteins and display similar β-1 -integrin properties. *Yersinia enterocolitica* additionally provides a gene referred to as ail (attachment-invasion-locus) encoding a 17-kDa protein known as the ail protein and which, in addition to contributing to adherence and invasion, is primarily responsible for serum resistance (Wachtel & Miller, Infect. Immunity 1995, 63 (7), 2541-2548). Both genes are found not to be homologous. *Yersinia pestis* also has an adhesion protein, encoded by a gene which

is highly homologous to Yersinia pseudotuberculosis Inv protein gene, but which is usually non functional as the gene is interrupted by an IS200-like element.

Work done on *Y. pseudotuberculosis* invasin, a 986-residue protein, showed that the C-terminal 192 amino acids are essential for binding of β-1 integrin receptor. However, to be most effective, uptake into mammalian cells requires also the presence of a self-association domain located N-terminal to the cell adhesion domain, which promotes invasin multimerization. Interaction of homomultimeric invasin with multiple integrins would establish tight adherence and receptor clustering, thus providing a signal for internalization (Dersch and Isberg, EMBO J, 18, 1199-213, 1999). On the other hand, invasin is not involved in the systemic dissemination of yersiniae (Pepe and Miller, Proc Natl Acad Sci, 90, 6473-7, 1993).

Other invasins are known such as Shigella invasin but they are very different from Yersinia invasin both structurally and functionally. *Escherichia coli* adhesion protein, known as intimin, is structurally related to Yersinia invasin but it has a different receptor: *Escherichia coli* translocates its receptor for intimin – the Tir protein – into the host cell.

US patent 5.338.842 discloses Yersinia inv nucleic acids. Young et al. (Mol Microbiol 1990 Jul;4(7):1119-28) describe *Yersinia enterocolitica* protein and nucleotide sequences, and *Yersinia pseudotuberculosis* protein sequence. Isberg et al. (Cell 50, 1987, 769-778) disclose *Yersinia pseudotuberculosis* nucleotide sequence. WO 00/23462 discloses a method for the production of purified invasin protein and uses thereof. WO 00/15175 relates to the use of molecules binding to β1-integrins and with the capacity to kill cells having β1-integrins for the production of pharmaceuticals.

Apart from bypassing the requirement for painful injections and the associated negative effect on patient compliance because of "needle fear", mucosal vaccination is attractive since it has been shown in animals that mucosal administration of antigens has a greater efficiency of inducing protective responses at mucosal surfaces, which is the route of entry of many pathogens. In addition, it has been suggested that mucosal vaccination, such as intranasal vaccination, may induce mucosal immunity not only in the nasal mucosa, but also in distant mucosal sites

such as the genital mucosa (Mestecky, 1987, Journal of Clinical Immunology, 7, 265-276; McGhee and Kiyono, Infectious Agents and Disease, 1993, 2, 55-73). More advantageously, besides its superiority in inducing mucosal immune responses, one attractive advantage of the mucosal vaccination relies on its ability to also induce good systemic immunity. The non-parenteral administration of vaccines may therefore be an efficient and more convenient way to boost systemic immunity induced by parenteral vaccination, especially when multiple boosts are required to sustain a vigourous systemic immunity.

Despite much research in the field, non-toxic and effective adjuvants which are suitable for use in humans, remains to be identified. The present invention provides a solution to this problem.

The present invention relates to the surprising finding that Yersinia adhesion proteins such as Invasin (Inv) protein from *Y. pseudotuberculosis* or *Yersinia enterocolitica,* or the "ail" protein from *Yersinia enterocolitica,* act as potent mucosal adjuvants in inducing or boosting immune responses to co-administered antigens. The Yersinia adhesion protein adjuvants when formulated with an antigen into a vaccine may be administered mucosally to a patient, and induce antigen specific immune responses which are at least as good as the immune response induced by a parenteral administration of the same vaccine. Advantageously, Yersinia adhesion proteins used as an adjuvant may induce local immunity, but also systemic immune responses and also other mucosal immune responses at distant mucosal sites. The immune responses induced by mucosal administration of vaccines of the present invention are at least as high as and preferably greater than those observed after a systemic injection of conventional vaccine.

The present invention provides safe and potent adjuvants which are easy to manufacture, which may be administered for vaccine purposes to humans, either through the mucosal route or the conventional parenteral (systemic) route. In particular the invention provides an adjuvant composition comprising an adhesion protein from Yersinia genus, preferably an invasin protein or an ail protein from the Yersinia genus. In another embodiment the adjuvant composition of the invention may comprise more than one, preferably at least two Yersinia adhesion proteins.

The adjuvants according to the present invention can be any fragment, variant or peptide of the adhesion proteins from *Y. pseudotuberculosis* or *Yersinia enterocolitica*, preferably any fragment, variant or peptide of the Inv or the ail protein, and will be therefrom referred to as "Inv or ail protein". The term fragment encompasses such fragments which typically comprise 500 or fewer amino acids, preferably fewer than 400, preferably fewer than 300, frequently containing as few as 200 amino acids or less. The term variant encompasses any deletion, insertion, substitution of amino acids in the Inv or ail protein, as long as it has retained its properties to bind its receptor. Fusion proteins comprising one or more variants, peptides or fragments from Yersinia Inv or ail protein are also within the frame of the invention. Preferably said Inv or ail protein is highly purified or solubilised from Yersinia, but the Inv or ail protein can also be produced using recombinant DNA technology. More preferably the adhesion protein is from *Yersinia pseudotuberculosis* or from *Yersinia enterocolitica*, yet most preferably from *Yersinia enterocolitica*. In a preferred embodiment, the adhesion protein is the gene product of the *Yersinia enterocolitica* inv gene or a fragment thereof. In a another preferred embodiment, the adhesion protein is the gene product of the *Yersinia pseudotuberculosis* inv gene or a fragment thereof.

Accordingly, as used herein a preferred embodiment utilises the *Yersinia enterocolitica* 835 residues of Inv protein, more preferably the carboxy-terminal 397 carboxy-terlminal residues on the Inv protein, yet most preferably the carboxy-terminal 195 residues of Inv protein, which is advantageous in that they can be expressed as a soluble protein and that they bind integrins and promote antigen uptake when attached to bacteria or beads. Another aspect of the invention is embodied by the use of *Yersinia pseudotuberculosis* 986-residue Inv protein, a particularly preferred form include the carboxy-terminal half of the protein, and a most preferred form include the C-terminal part of the protein having retained its self-association domain.

Optionally the adjuvant composition may further comprise a pharmaceutically acceptable carrier. A carrier molecule, within the definition of the invention, encompasses several forms, with the proviso that it is not a carrier organism such as

a live bacterial vector or a bacterial carrier strain. More particularly, a carrier molecule according to the present invention may include a porous polymeric particle, such as a microbead or a nanoparticle, and a metallic salt particle such as aluminium hydroxide, aluminium phosphate or calcium phosphate, or magnesium phosphate, iron phosphate, calcium carbonate, magnesium carbonate, calcium sufate, magnesium hydroxyde, or double salts like ammonium-iron phosphate, potassium-iron phosphate, calcium-iron phosphate, calcium-magnesium carbonate, or a mix of any of those salts. Alternatively the adjuvant composition may be provided within vehicle structures. The adjuvant of the invention may be provided either soluble in a liquid form or in a lyophilised form. The adjuvant of the invention may be presented in a slow release, a delayed-release or a quick release formulation. Accordingly, the preferred adjuvant composition in the present invention is *Yersinia enterocolitica* 835 residues of Inv protein, more preferably the carboxy-terminal 397 carboxy-terlminal residues on the Inv protein, yet most preferably the carboxy-terminal 195 residues of Inv protein, in the presence of a carrier, preferably a metallic salt particle, more preferably aluminium hydroxide, calcium phosphate or iron phosphate. Another preferred adjuvant composition according to the invention is *Yersinia pseudotuberculosis* 986-residue Inv protein, more particularly the carboxy-terminal half of the protein, most preferably the C-terminal part of the protein, in the absence of a carrier.

These formulations are potent adjuvants, also exhibit a good safety profile and are well tolerated by patients.

In another embodiment of the invention, the adhesion protein of the invention is a fusion protein wherein the Inv or ail protein is linked to a heterologous partner. The fusion partner may enhance the immunogenicity of heterologous immunogenic polypeptides with which it is fused. Alternatively the fusion partner may allow the expression at high levels of the fusion protein, or the production of a fusion protein which is and remains soluble throughout the purification process. The two moities within the fusion may be chemically conjugated, or alternatively preferably expressed as recombinant fusion proteins. Briefly, DNA sequences encoding the polypeptide components may be assembled separately, and ligated into an

appropriate expression vector. The 3' end of the DNA sequence encoding one polypeptide component is ligated, with or without a peptide linker, to the 5' end of a DNA sequence encoding the second polypeptide component so that the reading frames of the sequences are in phase. This permits translation into a single fusion polypeptide that retains the biological activity of both component polypeptides. A fusion partner may alternatively assist in providing T helper epitopes (an immunological fusion partner), preferably T helper epitopes recognized by humans, or may assist in expressing the protein (an expression enhancer) allowing increased levels to be produced in an heterologous expression system as compared to the non-fused Inv protein. Certain preferred fusion partners are both immunological and expression enhancing fusion partners. Other fusion partners may be selected so as to increase the solubility of the Inv protein or to enable the Inv protein to be targeted to desired intracellular compartments. Still further fusion partners include affinity tags, which facilitate purification of the polypeptide.

The fusion polypeptide can comprise a polypeptide as described herein together with an unrelated immunogenic protein, such as an immunogenic protein capable of eliciting a recall response. Examples of such proteins include tetanus, tuberculosis and hepatitis proteins (*see*, for example, Stoute et al. *New Engl. J. Med.*, *336*:86-91, 1997).

In one preferred embodiment, the immunological fusion partner is derived from a Mycobacterium sp., such as a Mycobacterium tuberculosis-derived Ra12 fragment. Briefly, Ra12 refers to a 14 KD C-terminal fragment corresponding to amino acid residues 192 to 323 of MTB32A. MTB32A is a serine protease of 32 KD molecular weight encoded by a gene in virulent and avirulent strains of *M. tuberculosis*. The nucleotide sequence and amino acid sequence of MTB32A have been described (for example, Skeiky *et al.*, *Infection and Immun.* (1999) 67:3998-4007 and also in WO 99/09186). Other preferred Ra12 polynucleotides generally comprise at least about 15 consecutive nucleotides, at least about 30 nucleotides, at least about 60 nucleotides, at least about 100 nucleotides, at least about 200 nucleotides, or at least about 300 nucleotides that encode a portion of a Ra12 polypeptide. Ra12 polynucleotides may comprise a native sequence (*i.e.*, an

endogenous sequence that encodes a Ra12 polypeptide or a portion thereof) or may comprise a variant of such a sequence. Ra12 polynucleotide variants may contain one or more substitutions, additions, deletions and/or insertions such that the biological activity of the encoded fusion polypeptide is not substantially diminished, relative to a fusion polypeptide comprising a native Ra12 polypeptide. Variants preferably exhibit at least about 70% identity, more preferably at least about 80% identity and most preferably at least about 90% identity to a polynucleotide sequence that encodes a native Ra12 polypeptide or a portion thereof.

Within other preferred embodiments, an immunological fusion partner is derived from protein D, a surface protein of the gram-negative bacterium Haemophilus influenza B (WO 91/18926). Preferably, a protein D derivative comprises approximately the first third of the protein (*e.g.*, the first N-terminal 100-110 amino acids), and a protein D derivative may be lipidated. Within certain preferred embodiments, the first 109 residues of a Lipoprotein D fusion partner is included on the N-terminus to provide the polypeptide with additional exogenous T-cell epitopes and to increase the expression level in *E. coli* (thus functioning as an expression enhancer). The lipid tail ensures optimal presentation of the antigen to antigen presenting cells. Other fusion partners include the non-structural protein from influenzae virus, NS1 (hemaglutinin). Typically, the N-terminal 81 amino acids are used, although different fragments that include T-helper epitopes may be used.

In another embodiment, the immunological fusion partner is the protein known as LYTA, or a portion thereof (preferably a C-terminal portion). LYTA is derived from *Streptococcus pneumoniae*, which synthesizes an N-acetyl-L-alanine amidase known as amidase LYTA (encoded by the LytA gene; *Gene 43*:265-292, 1986). LYTA is an autolysin that specifically degrades certain bonds in the peptidoglycan backbone. The C-terminal domain of the LYTA protein is responsible for the affinity to the choline or to some choline analogues such as DEAE. This property has been exploited for the development of *E. coli* C-LYTA expressing plasmids useful for expression of fusion proteins. Purification of hybrid proteins containing the C-LYTA fragment at the amino terminus has been described (*see Biotechnology*

10:795-798, 1992). Within a preferred embodiment, a repeat portion of LYTA may be incorporated into a fusion polypeptide. A repeat portion is found in the C-terminal region starting at residue 178. A particularly preferred repeat portion incorporates residues 188-305.

Yet in another embodiment an immunological fusion partner is derived from the Glutathione S-transferase (GST), a naturally occurrig 26kDa protein which can be expressed in *E. coli* with full enzymatic activity. Fusion proteins which possess the complete amino acid sequence of GST can undergo dimerisation and can be purified easily from bacterial lysates by affinity chromatography. Preferably the recombinant strategy includes cloning a gene construct encoding a GST (glutathione S-transferase) fusion protein, the gene construct comprising from 5' to 3' a DNA sequence encoding GST joined to a DNA sequence encoding the adhesion protein, into an expression vector to form a DNA fragment encoding a GST- carboxyl-terminal invasin fusion protein. An affinity polyhistidine tail may be engineered at the carboxy-terminus of the fusion protein allowing for simplified purification through affinity chromatography.

One embodiment of the present invention consists of a vaccine formulation comprising an adhesion protein such as Yersinia Inv or ail protein, and an antigen or antigenic composition. Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A. 1978.

The Yersinia adhesion protein may be admixed to the antigen in the vaccine formulation or alternatively linked to the antigen through a direct or indirect linkage. Given the described function of Yersinia adhesion proteins in the art, surprisingly a preferred vaccine formulation according to the invention is where the antigen or antigenic composition is not linked to the adhesion protein through a direct linkage (such as a covalent linkage or conjugation) or indirect linkage or association. In this situation it is particularly preferred that the Yersinia adhesion protein is *Yersinia pseudotuberculosis* Inv protein invasin, more preferably the C-terminal half of the protein having retained its self-association capability. Such

formulations where antigen is merely admixed with the Yersinia adjuvant are advantageous in many respects including ease of vaccine formulation.

In other embodiments of the present invention the Yersinia adhesion protein and the vaccine antigen are associated directly through covalent linkage such as by chemical conjugation (see Chemistry of protein conjugation and cross-linking, S.S. Wong (1991) CRC Press) or fusion, or indirectly through co-adsorption onto a carrier molecule such as a liposome or a metallic salt particle.

In another embodiment of the present invention the Yersinia adhesion protein may be adsorbed onto one metallic salt particle and the antigen may be adsorbed onto a different metallic salt particle, the vaccine being formulated by admixture of the two adsorbed metallic salt complexes.

Additionally the vaccine composition may further comprise a pharmaceutically acceptable carrier, an excipient or a diluent. Examples of suitable pharmaceutically acceptable excipients for use in the combinations of the present invention include water, phosphate buffered saline, isotonic buffer solutions.

The adhesion protein:antigen ratio should be in the ratio of 1:1000 and 1000:1, preferably from 1:100 to 100:1 and most preferably from 1:1. Preferably the adhesion protein in the vaccine according to the invention is a purified recombinantly produced Inv protein from *Yersinia pseudotuberculosis* or Inv or ail protein from *Yersinia enterocolitica*. More preferably the adhesion protein in the vaccine according to the invention includes the 835 residues of the *Yersinia enterocolitica* Inv protein, more preferably the carboxy-terminal 397 carboxy-terlminal residues of the *Yersinia enterocolitica* Inv protein, yet most preferably the adhesion protein includes the carboxy-terminal 195 residues of Inv protein. Alternatively, the adhesion protein in the vaccine of the invention includes the recombinantly produced *Yersinia pseudotuberculosis* Inv protein invasin, and more preferably includes the 986-residue protein, ideally the carboxy-terminal half of the protein and most preferably the C-terminal half of the protein having retained its self-association capability.

The adjuvant combinations of the present invention may be used as both systemic or mucosal adjuvant. In a particular form of the invention there is provided

a systemic vaccine to be administered through the systemic or parenteral route such as intramuscular, intradermal, transdermal, subcutaneous, intraperitoneal or intravenous administration. In a preferred embodiment of the present invention there is provided an adjuvant for use in mucosal vaccine compositions. Such adjuvants are well tolerated in humans and are potent in their induction or boost of systemic immune responses. The adjuvants of the present invention may take various forms depending on the administration route.

A preferred route of administration of the vaccine is via the intranasal route, for example by a spray or a droplet, or through the pulmonary route, or through the buccal route or the oral route, for example by a gastro-resistant granule, a capsule or a lyophilised tablet. Of these routes, the oral route is particularly preferred. In such cases the pharmaceutically acceptable excipient may also include alkaline buffers, or enteric capsules or microgranules. The vaccines of the present invention may also be administered by the vaginal route. In such cases, the pharmaceutically acceptable excipients may also include emulsifiers, polymers such as CARBOPOL®, and other known stabilisers of vaginal creams and suppositories. The vaccines of the present invention may also be administered by the rectal route. In such cases the excipients may also include waxes and polymers known in the art for forming rectal suppositories.

Accordingly, the vaccine preparations of the present invention may be used to protect or treat a mammal susceptible to, or suffering from disease, by means of administering said vaccine via the systemic route, such as intramuscular, intradermal, transdermal, subcutaneous, intraperitoneal or intravenous, or more preferably via a mucosal route, such as the oral/bucal/intestinal/vaginal/rectal or nasal route. Such intranasal administration may be in a droplet, spray, or dry powdered form. Nebulised or aerosolised vaccine formulations also form part of this invention. Enteric formulations such as gastro resistant capsules and granules for oral administration, suppositories for rectal or vaginal administration, and skin patches for transdermal or transcutaneous delivery also form part of this invention. The vaccine formulations of the present invention may then be applied to a mucosal surface of a mammal in either a priming or boosting vaccination regime; or

alternatively be administered systemically, for example *via* the transdermal, subcutaneous, intradermal, intraperitoneal, intravenous or intramuscular routes. A preferred route of administration is via the mucosal route, for example by intranasal or oral route.

The present invention also relates to the use of a Yersinia adhesion protein adjuvant, preferably the Inv or ail proteins to induce or boost immune responses to co-administered antigens. More than one, preferably two different Yersinia adhesion proteins may be associated with the antigen. The antigen may be admixed with the adjuvant, and consequently administered in free solution together with free Inv or ail protein adjuvant. In this situation it is particularly preferred that the Yersinia adhesion protein is *Yersinia pseudotuberculosis* Inv protein invasin, more preferably the C-terminal half of the protein having retained its self-association capability. In such a formulation the antigen is not associated, i.e. linked, attached or conjugated with the adjuvant.

Methods of systemic administration of the vaccine preparations may include conventional syringes and needles, or devices designed for ballistic delivery of solid vaccines (WO 99/27961), or needleless pressure liquid jet device (US 4,596,556; US 5,993,412), or transdermal patches (WO 97/48440; WO 98/28037). The present invention may also be used to enhance the immunogenicity of antigens applied to the skin (transdermal or transcutaneous delivery WO 98/20734 ; WO 98/28037). The present invention therefore provides a delivery device for systemic administration, pre-filled with the vaccine or adjuvant compositions of the present invention.

Adjuvant and vaccine preparations containing an additional adjuvant other than the Yersinia adhesion proteins also form part of the present invention. For example combinations of the Inv or ail protein with at least one of the following group comprising QS21, 3D-MPL, a polyoxyethylene ether or ester, or CpG are encompassed within the scope of the invention.

3 De-O-acylated monophosphoryl lipid A is a well known adjuvant manufactured by Ribi Immunochem, Montana. It can be prepared by the methods taught in GB 2122204B. A preferred form of 3 De-O-acylated monophosphoryl lipid

A is in the form of an emulsion having a small particle size less than 0.2μm in diameter (EP 0 689 454 B1).

Immunostimulatory oligonucleotides containing unmethylated CpG dinucleotides ("CpG") and are known in the art as being adjuvants when administered by both systemic and mucosal routes (WO 96/02555, EP 468520, Davis *et al., J.Immunol*, 1998, 160(2):870-876; McCluskie and Davis, *J.Immunol.*, 1998, 161(9):4463-6). CpG is an abbreviation for cytosine-guanosine dinucleotide motifs present in DNA.

QS-21 is an haemolytic saponin is a HPLC purified fraction of Quil A (derived from the bark of the South American tree Quillaja Saponaria Molina) and is known as a potent adjuvant in vaccines for systemic and mucosal administration, and the method of its production is disclosed in US Patent No.5,057,540 and EP 0 362 279 B1.

Preferably the vaccine formulations of the present invention contain an antigen or antigenic composition capable of eliciting an immune response against a human pathogen, which antigen or antigenic composition is derived from HIV-1, (such as tat, nef, gp120 or gp160), human herpes viruses, such as gD or derivatives thereof or Immediate Early protein such as ICP27 from HSV1 or HSV2, cytomegalovirus ((esp Human)(such as gB or derivatives thereof), Rotavirus (including live-attenuated viruses), Epstein Barr virus (such as gp350 or derivatives thereof), Varicella Zoster Virus (such as gpI, II and IE63), or from a hepatitis virus such as hepatitis B virus (for example Hepatitis B Surface antigen or a derivative thereof), hepatitis A virus, hepatitis C virus and hepatitis E virus, or from other viral pathogens, such as paramyxoviruses: Respiratory Syncytial virus (such as F and G proteins or derivatives thereof), parainfluenza virus, measles virus, mumps virus, human papilloma viruses (for example HPV6, 11, 16, 18, ..), flaviviruses (e.g. Yellow Fever Virus, Dengue Virus, Tick-borne encephalitis virus, Japanese Encephalitis Virus) or Influenza virus (whole live or inactivated virus, split influenza virus, grown in eggs or MDCK cells, or whole flu virosomes (as described by R. Gluck, Vaccine, 1992, 10, 915-920) or purified or recombinant proteins thereof, such as HA, NP, NA, or M proteins, or combinations thereof), or

derived from bacterial pathogens such as *Neisseria spp,* including *N. gonorrhea* and *N. meningitidis* (for example capsular polysaccharides and conjugates thereof, transferrin-binding proteins, lactoferrin binding proteins, PilC, adhesins); *S. pyogenes* (for example M proteins or fragments thereof, C5A protease, lipoteichoic acids), *S. agalactiae, S. mutans; H. ducreyi; Moraxella spp, including M catarrhalis, also known as Branhamella* catarrhalis (for example high and low molecular weight adhesins and invasins*); Bordetella spp, including B. pertussis* (for example pertactin, pertussis toxin or derivatives thereof, filamenteous hemagglutinin, adenylate cyclase, fimbriae), *B. parapertussis and B. bronchiseptica; Mycobacterium spp., including M. tuberculosis* (for example ESAT6, Antigen 85A, -B or –C), *M. bovis, M. leprae, M. avium, M. paratuberculosis, M. smegmatis; Legionella spp,* including *L. pneumophila; Escherichia spp,* including *enterotoxic E. coli* (for example colonization factors, heat-labile toxin or derivatives thereof, heat-stable toxin or derivatives thereof), enterohemorragic *E. coli,* enteropathogenic *E. coli* (for example shiga toxin-like toxin or derivatives thereof); *Vibrio spp,* including *V. cholera* (for example cholera toxin or derivatives thereof*); Shigella spp,* including *S. sonnei, S. dysenteriae, S. flexnerii; Yersinia spp,* including *Y.* enterocolitica (for example a Yop protein) , *Y. pestis, Y. pseudotuberculosis; Campylobacter spp,* including *C. jejuni* (for example toxins, adhesins, invasins and Campylobacter whole cells) and *C. coli; Salmonella spp,* including *S. typhi, S. paratyphi, S. choleraesuis, S. enteritidis; Listeria spp.,* including *L. monocytogenes; Helicobacter spp,* including *H.* pylori (for example urease, catalase, vacuolating toxin); *Pseudomonas spp,* including *P. aeruginosa; Staphylococcus spp.,* including *S. aureus, S. epidermidis; Enterococcus spp.,* including *E. faecalis, E. faecium; Clostridium spp.,* including *C.* tetani (for example tetanus toxin and derivative thereof), *C. botulinum* (for example botulinum toxin and derivative thereof*), C. difficile* (for example clostridium toxins A or B and derivatives thereof); *Bacillus spp.,* including *B. anthracis* (for example botulinum toxin and derivatives thereof*); Corynebacterium spp.,* including *C. diphtheriae* (for example diphtheria toxin and derivatives thereof); *Borrelia spp.,* including *B. burgdorferi* (for example OspA, OspC, DbpA, DbpB), *B. garinii* (for example

OspA, OspC, DbpA, DbpB), *B. afzelii* (for example OspA, OspC, DbpA, DbpB), *B. andersonii* (for example OspA, OspC, DbpA, DbpB), *B. hermsii; Ehrlichia spp.*, including *E. equi* and the agent of the Human Granulocytic Ehrlichiosis; *Rickettsia spp*, including *R. rickettsii; Chlamydia spp., including C. trachomatis* (for example MOMP, heparin-binding proteins), *C. pneumoniae* (for example MOMP, heparin-binding proteins), *C. psittaci; Leptospira spp.*, including *L. interrogans; Treponema spp.*, including *T. pallidum* (for example the rare outer membrane proteins), *T. denticola, T. hyodysenteriae;* or derived from parasites such as *Plasmodium spp.*, including *P. falciparum; Toxoplasma spp.*, including *T. gondii (for example SAG2, SAG3, Tg34); Entamoeba spp.*, including *E. histolytica; Babesia spp.*, including *B. microti; Trypanosoma spp.*, including *T. cruzi; Giardia spp.*, including *G. lamblia; Leshmania spp.*, including *L. major; Pneumocystis spp.*, including *P. carinii; Trichomonas spp.*, including *T. vaginalis; Schisostoma spp.*, including *S. mansoni*, or derived from yeast such as *Candida spp.*, including *C. albicans; Cryptococcus spp.*, including *C. neoformans*.

Other preferred specific antigens for *M. tuberculosis* are for example Tb Ra12, Tb H9, Tb Ra35, Tb38-1, Erd 14, DPV, MTI, MSL, mTTC2 and hTCC1 (WO 99/51748). Proteins for *M. tuberculosis* also include fusion proteins and variants thereof where at least two, preferably three polypeptides of *M. tuberculosis* are fused into a larger protein. Preferred fusions include Ra12-TbH9-Ra35, Erd14-DPV-MTI, DPV-MTI-MSL, Erd14-DPV-MTI-MSL-mTCC2, Erd14-DPV-MTI-MSL, DPV-MTI-MSL-mTCC2, TbH9-DPV-MTI (WO 99/51748).

Most preferred antigens for Chlamydia include for example the High Molecular Weight Protein (HWMP) (WO 99/17741), ORF3 (EP 366 412), and putative membrane proteins (Pmps). Other Chlamydia antigens of the vaccine formulation can be selected from the group described in WO 99/28475.

Examples of preferred *Moraxella catarrhalis* protein antigens (especially for the prevention of otitis media) are: OMP106 [WO 97/41731 (Antex) & WO 96/34960 (PMC)]; OMP21; LbpA &/or LbpB [WO 98/55606 (PMC)]; TbpA &/or TbpB [WO 97/13785 & WO 97/32980 (PMC)]; CopB [Helminen ME, *et al.* (1993) Infect. Immun. 61:2003-2010]; UspA1 and/or UspA2 [WO 93/03761 (University of

Texas)]; OmpCD; HasR (PCT/EP99/03824); PilQ (PCT/EP99/03823); OMP85 (PCT/EP00/01468); lipo06 (GB 9917977.2); lipo10 (GB 9918208.1); lipo11 (GB 9918302.2); lipo18 (GB 9918038.2); P6 (PCT/EP99/03038); D15 (PCT/EP99/03822); OmplA1 (PCT/EP99/06781); Hly3 (PCT/EP99/03257); and OmpE; FhaB (WO 99/58685).

Examples of non-typeable *Haemophilus influenzae* antigens (especially for the prevention of otitis media) include: Fimbrin protein [(US 5766608 - Ohio State Research Foundation)] and fusions comprising peptides therefrom [eg LB1(f) peptide fusions; US 5843464 (OSU) or WO 99/64067]; OMP26 [WO 97/01638 (Cortecs)]; P6 [EP 281673 (State University of New York)]; TbpA and/or TbpB; Hia; Hsf; Hin47; Hif; Hmw1; Hmw2; Hmw3; Hmw4; Hap; D15 (WO 94/12641); P2; and P5 (WO 94/26304); Iomp1457 (GB 0025493.8), YtfN (GB 0025488.8), VirG (GB 0026002.6), Iomp1681 (GB 0025998.6), OstA (GB 0025486.2).

Examples of Neisserial antigens (including gonococcus and meningococcus, (particularly *N. meningitidis* B) include: NspA (WO 96/29412), Hsf-like (WO 99/31132), Hap (PCT/EP99/02766), PorA, PorB, OMP85 (WO 00/23595), PilQ (PCT/EP99/03603), PldA (PCT/EP99/06718), FrpB (WO 96/31618), TbpA (US 5,912,336), TbpB, FrpA/FrpC (WO 92/01460), LbpA/LbpB (PCT/EP98/05117), FhaB (WO 98/02547), HasR (PCT/EP99/05989), lipo02 (PCT/EP99/08315), Tbp2 (WO 99/57280), MltA (WO 99/57280), and ctrA (PCT/EP00/00135).

Preferred bacterial vaccines comprise antigens derived from *Streptococcus spp,* including *S. pneumoniae*, for example capsular polysaccharides and conjugates thereof. Preferred *S. pneumoniae* antigens are those pneumococcal proteins which are exposed on the outer surface of the pneumococcus (capable of being recognised by a host's immune system during at least part of the life cycle of the pneumococcus), or are proteins which are secreted or released by the pneumococcus. Most preferably, the protein is a toxin, adhesin, 2-component signal tranducer, or lipoprotein of *S. pneumoniae*, or immunologically functional equivalents thereof. Particularly preferred proteins include but are not limited to: pneumolysin (preferably detoxified by chemical treatment or mutation) [WO 96/05859, WO 90/06951, WO 99/03884], PsaA and transmembrane deletion

variants thereof (Berry & Paton, Infect Immun 1996 Dec;64(12):5255-62), PspA and transmembrane deletion variants thereof (US 5804193, WO 92/14488, WO 99/53940), PspC and transmembrane deletion variants thereof (WO 97/09994, WO 99/53940), CbpA and transmembrane deletion variants thereof (WO 97/41151; WO 99/51266), Glyceraldehyde-3-phosphate – dehydrogenase (Infect. Immun. 1996 64:3544), HSP70 (WO 96/40928), PcpA (Sanchez-Beato et al. *FEMS Microbiol Lett* 1998, 164:207-14), M like protein (SB patent application No. EP 0837130), and adhesin 18627 (SB Patent application No. EP 0834568). The present invention also encompasses immunologically functional equivalents of such proteins (as defined above). The *S. pneumoniae* proteins used in the present invention are preferably selected from the group pneumolysin, PsaA, PspA, PspC, CbpA or a combination of two or more such proteins. The present invention also encompasses immunologically functional equivalents of such proteins (as defined above). Further preferred pneumococcal protein antigens are those disclosed in WO 98/18931, particularly those selected in WO 98/18930 and PCT/US99/30390. The *Streptococcus pneumoniae* protein of the invention is preferably selected from the group consisting of: a protein from the polyhistidine triad family (Pht), a choline binding protein, proteins having an LPXTG motif (where X is any amino acid), proteins having a Type II Signal sequence motif of LXXC (where X is any amino acid), and proteins having a Type I Signal sequence motif. Preferred examples within these categories (or motifs) are the following proteins (or immunologically functional equivalent thereof): PhtA is disclosed in WO 98/18930, and is also called Sp36. It is a protein from the polyhistidine triad family and has the type II signal motif of LXXC; PhtD is disclosed in WO 00/37105, and is also called Sp036D. It also is a protein from the polyhistidine triad family and has the type II LXXC signal motif; PhtB is disclosed in WO 00/37105, and is also called Sp036B. Another member of the PhtB family is the C3-Degrading Polypeptide, as disclosed in WO 00/17370. This protein also is from the polyhistidine triad family and has the type II LXXC signal motif. A preferred immunologically functional equivalent is the protein Sp42 disclosed in WO 98/18930; SpsA is a Choline binding protein disclosed in WO 98/39450; LytB is a Choline binding protein disclosed in WO

98/18930, and is also called Sp46; LytC is a Choline binding protein disclosed in WO 98/18930, and is also called Sp91; Sp125 is an example of a pneumococcal surface protein with the Cell Wall Anchored motif of LPXTG (where X is any amino acid). Sp125 itself is disclosed in WO 98/18930, and is also known as ZmpB – a zinc metalloproteinase. Sp101 is disclosed in WO 98/06734 (where it has the reference # y85993. It is characterised by a Type I signal sequence. Sp133 is disclosed in WO 98/06734 (where it has the reference # y85992. It is also characterised by a Type I signal sequence. The proteins used in the present invention are preferably selected from the group PhtD and PhtA, or a combination of both of these proteins.

Other preferred bacterial vaccines comprise antigens derived from *Haemophilus spp., including H. influenzae type B* (for example PRP and conjugates thereof), *non typeable H. influenzae,* for example OMP26, high molecular weight adhesins, P5, P6, protein D and lipoprotein D, and fimbrin and fimbrin derived peptides (US 5,843,464) or multiple copy varients or fusion proteins thereof.

Derivatives of Hepatitis B Surface antigen are well known in the art and include, inter alia, those PreS1, PreS2 S antigens set forth described in European Patent applications EP-A-414 374; EP-A-0304 578, and EP 198-474. In one preferred aspect the vaccine formulation of the invention comprises the HIV-1 antigen, gp120, especially when expressed in CHO cells. In a further embodiment, the vaccine formulation of the invention comprises gD2t as hereinabove defined.

In a preferred embodiment of the present invention vaccines containing the claimed adjuvant comprise antigen derived from the Human Papilloma Virus (HPV) considered to be responsible for genital warts (HPV 6 or HPV 11 and others), and the HPV viruses responsible for cervical cancer (HPV16, HPV18 and others).

Particularly preferred forms of genital wart prophylactic, or therapeutic, vaccine comprise L1 particles or capsomers, and fusion proteins comprising one or more antigens selected from the HPV 6 and HPV 11 proteins E6, E7, L1, and L2.

The most preferred forms of fusion protein are: L2E7 as disclosed in WO 96/26277, and proteinD(1/3)-E7 disclosed in GB 9717953.5 (PCT/EP98/05285).

A preferred HPV cervical infection or cancer, prophylaxis or therapeutic vaccine, composition may comprise HPV 16 or 18 antigens. For example, L1 or L2 antigen monomers, or L1 or L2 antigens presented together as a virus like particle (VLP) or the L1 alone protein presented alone in a VLP or caposmer structure. Such antigens, virus like particles and capsomer are per se known. See for example WO94/00152, WO94/20137, WO94/05792, and WO93/02184.

Additional early proteins may be included alone or as fusion proteins such as E7, E2 or preferably E5 for example; particularly preferred embodiments of this includes a VLP comprising L1E7 fusion proteins (WO 96/11272).

Particularly preferred HPV 16 antigens comprise the early proteins E6 or E7 in fusion with a protein D carrier to form Protein D - E6 or E7 fusions from HPV 16, or combinations thereof; or combinations of E6 or E7 with L2 (WO 96/26277).

Alternatively the HPV 16 or 18 early proteins E6 and E7, may be presented in a single molecule, preferably a Protein D- E6/E7 fusion. Such vaccine may optionally contain either or both E6 and E7 proteins from HPV 18, preferably in the form of a Protein D - E6 or Protein D - E7 fusion protein or Protein D E6/E7 fusion protein.

The vaccine of the present invention may additionally comprise antigens from other HPV strains, preferably from strains HPV 31 or 33.

Vaccines of the present invention further comprise antigens derived from parasites that cause Malaria. For example, preferred antigens from *Plasmodia falciparum* include RTS,S and TRAP. RTS is a hybrid protein comprising substantially all the C-terminal portion of the circumsporozoite (CS) protein of *P.falciparum* linked via four amino acids of the preS2 portion of Hepatitis B surface antigen to the surface (S) antigen of hepatitis B virus. It's full structure is disclosed in the International Patent Application No. PCT/EP92/02591, published under Number WO 93/10152 claiming priority from UK patent application No.9124390.7. When expressed in yeast RTS is produced as a lipoprotein particle, and when it is co-expressed with the S antigen from HBV it produces a mixed particle known as RTS,S. TRAP antigens are described in the International Patent Application No. PCT/GB89/00895, published under WO 90/01496. A preferred embodiment of the

present invention is a Malaria vaccine wherein the antigenic preparation comprises a combination of the RTS,S and TRAP antigens. Other plasmodia antigens that are likely candidates to be components of a multistage Malaria vaccine are *P. faciparum* MSP1, AMA1, MSP3, EBA, GLURP, RAP1, RAP2, Sequestrin, PfEMP1, Pf332, LSA1, LSA3, STARP, SALSA, PfEXP1, Pfs25, Pfs28, PFS27/25, Pfs16, Pfs48/45, Pfs230 and their analogues in Plasmodium spp.

It is a particularly preferred aspect of the present invention that the vaccines comprise a tumour antigen, such vaccines are surprisingly potent in the immunotherapy of cancer such as prostrate, breast, colorectal, lung, pancreatic, renal, ovarian or melanoma cancers. Accordingly, the formulations may contain tumour-associated antigen, as well as antigens associated with tumour-support mechanisms (e.g. angiogenesis, tumour invasion). Exemplary antigens include MAGE 1 and MAGE 3 or other MAGE antigens (for the treatment of melanoma), PRAME, BAGE, or GAGE (Robbins and Kawakami, 1996, Current Opinions in Immunology 8, pps 628-636; Van den Eynde et al., International Journal of Clinical & Laboratory Research (submitted 1997); Correale et al. (1997), Journal of the National Cancer Institute 89, p293. Indeed these antigens are expressed in a wide range of tumour types such as melanoma, lung carcinoma, sarcoma and bladder carcinoma. Other tumour-specific antigens are suitable for use with the adjuvants of the present invention and include, but are not restricted to tumour-specific gangliosides, Prostate specific antigen (PSA) or Her-2/neu, KSA (GA733), PAP, mammaglobin, MUC-1, carcinoembryonic antigen (CEA), Cripto antigen. Additionally, antigens particularly relevant for vaccines in the therapy of cancer also comprise Prostate-specific membrane antigen (PSMA), Prostate Stem Cell Antigen (PSCA), tyrosinase, survivin, NY-ESO1, prostase, PS108 (WO 98/50567), RAGE, LAGE, HAGE. Additionally said antigen may be a self peptide hormone such as whole length Gonadotrophin hormone releasing hormone (GnRH, WO 95/20600), a short 10 amino acid long peptide, useful in the treatment of many cancers, or in immunocastration. Accordingly in one aspect of the present invention there is provided a vaccine comprising an adjuvant composition according to the invention and a tumour rejection antigen.

It is foreseen that compositions of the present invention will be used to formulate vaccines containing antigens derived from *Borrelia sp.*. For example, antigens may include nucleic acid, pathogen derived antigen or antigenic preparations, recombinantly produced protein or peptides, and chimeric fusion proteins. In particular the antigen is OspA. The OspA may be a full mature protein in a lipidated form virtue of the host cell (E.Coli) termed (Lipo-OspA) or a non-lipidated derivative. Such non-lipidated derivatives include the non-lipidated NS1-OspA fusion protein which has the first 81 N-terminal amino acids of the non-structural protein (NS1) of the influenza virus, and the complete OspA protein, and another, MDP-OspA is a non-lipidated form of OspA carrying 3 additional N-terminal amino acids.

Vaccines of the present invention may be used for the prophylaxis or therapy of allergy. Such vaccines would comprise allergen specific (for example Der p1) and allergen non-specific antigens (for example peptides derived from human IgE, including but not restricted to the stanworth decapeptide (EP 0 477 231 B1)).

Vaccines of the present invention may also be used for the prophylaxis or therapy of chronic disorders others than allergy, cancer or infectious diseases. Such chronic disorders are diseases such as atherosclerosis, and Alzheimer.

Antigens relevant for the prophylaxis and the therapy of patients susceptible to or suffering from Alzheimer neurodegenerative disease are, in particular, the N terminal 39 –43 amino acid fragment (Aβ) of the amyloid precursor protein and smaller fragments. This antigen is disclosed in the International Patent Application No. WO 99/27944 – (Athena Neurosciences).

The adhesion proteins and antigens of the present invention may be expressed in an appropriate host cell, and preferably in *E. coli*. In a preferred embodiment the proteins are expressed with an affinity tag, such as for example, a histidine tail comprising between 5 to 9 and preferably six histidine residues, most preferably at least 4 histidine residues. These are advantageous in aiding purification through for example ion metal affinity chromatography (IMAC).

The present invention also provides a nucleic acid encoding the adhesion proteins and antigens of the present invention. Such sequences can be inserted into

a suitable expression vector and used for DNA/RNA vaccination or expressed in a suitable host. In additional embodiments, genetic constructs comprising one or more of the polynucleotides of the invention are introduced into cells *in vivo*. This may be achieved using any of a variety or well-known approaches. One of the preferred methods for *in vivo* delivery of one or more nucleic acid sequences involves the use of an expression vector such as a recombinant live viral or bacterial microorganism. Suitable viral expression vectors are for example poxviruses (e.g; vaccinia, fowlpox, canarypox), alphaviruses (Sindbis virus, Semliki Forest Virus, Venezuelian Equine Encephalitis Virus), adenoviruses, adeno-associated virus, picornaviruses (poliovirus, rhinovirus), and herpesviruses (varicella zoster virus, etc). Other preferred methods for *in vivo* delivery of one or more nucleic acid sequences involves the use of a bacterial expression vector, such as Listeria, Salmonella , Shigella and BCG. Inoculation and in vivo infection with this live vector will lead to in vivo expression of the antigen and induction of immune responses. These viruses and bacteria can be virulent, or attenuated in various ways in order to obtain live vaccines. Such live vaccines also form part of the invention.

A DNA sequence encoding the adhesion proteins and antigens of the present invention can be synthesized using standard DNA synthesis techniques, such as by enzymatic ligation as described by D.M. Roberts *et al.* in Biochemistry 1985, *24*, 5090-5098, by chemical synthesis, by *in vitro* enzymatic polymerization, or by PCR technology utilising for example a heat stable polymerase, or by a combination of these techniques. Enzymatic polymerisation of DNA may be carried out *in vitro* using a DNA polymerase such as DNA polymerase I (Klenow fragment) in an appropriate buffer containing the nucleoside triphosphates dATP, dCTP, dGTP and dTTP as required at a temperature of 10°-37°C, generally in a volume of 50μl or less. Enzymatic ligation of DNA fragments may be carried out using a DNA ligase such as T4 DNA ligase in an appropriate buffer, such as 0.05M Tris (pH 7.4), 0.01M $MgCl_2$, 0.01M dithiothreitol, 1mM spermidine, 1mM ATP and 0.1mg/ml bovine serum albumin, at a temperature of 4°C to ambient, generally in a volume of 50ml or less. The chemical synthesis of the DNA polymer or fragments may be carried out by conventional phosphotriester, phosphite or phosphoramidite

chemistry, using solid phase techniques such as those described in 'Chemical and Enzymatic Synthesis of Gene Fragments - A Laboratory Manual' (ed. H.G. Gassen and A. Lang), Verlag Chemie, Weinheim (1982), or in other scientific publications, for example M.J. Gait, H.W.D. Matthes, M. Singh, B.S. Sproat, and R.C. Titmas, Nucleic Acids Research, 1982, 10, 6243; B.S. Sproat, and W. Bannwarth, Tetrahedron Letters, 1983, 24, 5771; M.D. Matteucci and M.H. Caruthers, Tetrahedron Letters, 1980, 21, 719; M.D. Matteucci and M.H. Caruthers, Journal of the American Chemical Society, 1981, 103, 3185; S.P. Adams *et al.*, Journal of the American Chemical Society, 1983, 105, 661; N.D. Sinha, J. Biernat, J. McMannus, and H. Koester, Nucleic Acids Research, 1984, 12, 4539; and H.W.D. Matthes *et al.*, EMBO Journal, 1984, 3, 801.

In a further embodiment of the invention is provided a method of producing a adhesion proteins and antigens as described herein. The process of the invention may be performed by conventional recombinant techniques such as described in Maniatis *et al.*, Molecular Cloning - A Laboratory Manual; Cold Spring Harbor, 1982-1989.

In particular, the process of the invention may preferably comprise the steps of:

i) preparing a replicable or integrating expression vector capable, in a host cell, of expressing a DNA polymer comprising a nucleotide sequence that encodes the protein or an immunogenic derivative thereof;

ii) transforming a host cell with said vector;

ii) culturing said transformed host cell under conditions permitting expression of said DNA polymer to produce said protein; and

iv) recovering said protein.

The term 'transforming' is used herein to mean the introduction of foreign DNA into a host cell. This can be achieved for example by transformation, transfection or infection with an appropriate plasmid or viral vector using e.g. conventional techniques as described in Genetic Engineering; Eds. S.M. Kingsman and A.J. Kingsman; Blackwell Scientific Publications; Oxford, England, 1988. The

term 'transformed' or 'transformant' will hereafter apply to the resulting host cell containing and expressing the foreign gene of interest. Preferably recombinant adhesion proteins and antigens of the invention are expressed in unicellular hosts, most preferably in bacterial systems, most preferably in *E. coli*.

The expression vectors are novel and also form part of the invention.

The replicable expression vectors may be prepared in accordance with the invention, by cleaving a vector compatible with the host cell to provide a linear DNA segment having an intact replicon, and combining said linear segment with one or more DNA molecules which, together with said linear segment encode the desired product, such as the DNA polymer encoding the protein of the invention, or derivative thereof, under ligating conditions.

Thus, the hybrid DNA may be pre-formed or formed during the construction of the vector, as desired.

The choice of vector will be determined in part by the host cell, which may be prokaryotic or eukaryotic but are preferably *E. coli*, yeast or CHO cells. Suitable vectors include plasmids, bacteriophages, cosmids and recombinant viruses. Expression and cloning vectors preferably contain a selectable marker such that only the host cells expressing the marker will survive under selective conditions. Selection genes include but are not limited to the one encoding protein that confer a resistance to ampicillin, tetracyclin or kanamycin. Expression vectors also contain control sequences which are compatible with the designated host. For example, expression control sequences for *E. coli*, and more generally for prokaryotes, include promoters and ribosome binding sites. Promoter sequences may be naturally occurring, such as the -lactamase (penicillinase) (Weissman 1981, *In Interferon 3* (ed. L. Gresser), lactose (lac) (Chang et al. Nature, 1977, 198: 1056) and tryptophan (trp) (Goeddel et al. Nucl. Acids Res. 1980, 8, 4057) and lambda-derived P_L promoter system. In addition, synthetic promoters which do not occur in nature also function as bacterial promoters. This is the case for example for the tac synthetic hybrid promoter which is derived from sequences of the trp and lac promoters (De Boer et al., Proc. Natl Acad Sci. USA 1983, 80, 21-26). These systems are particularly suitable with *E. coli*.

Yeast compatible vectors also carry markers that allow the selection of successful transformants by conferring prototrophy to auxotrophic mutants or resistance to heavy metals on wild-type strains. Control sequences for yeast vectors include promoters for glycolytic enzymes (Hess et al., J. Adv. Enzyme Reg. 1968, 7, 149), PHO5 gene encoding acid phosphatase, CUP1 gene, ARG3 gene, GAL genes promoters and synthetic promoter sequences.. Other control elements useful in yeast expression are terminators and leader sequences. The leader sequence is particularly useful since it typically encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell. Suitable signal sequences can be encoded by genes for secreted yeast proteins such as the yeast invertase gene and the a-factor gene, acid phosphatase, killer toxin, the a-mating factor gene and recently the heterologous inulinase signal sequence derived from INU1A gene of Kluyveromyces marxianus.. Suitable vectors have been developed for expression in *Pichia pastoris* and *Saccharomyces cerevisiae*.

A variety of *P. pastoris* expression vectors are available based on various inducible or constitutive promoters (Cereghino and Cregg, FEMS Microbiol. Rev. 2000,24:45-66). For the production of cytosolic and secreted proteins,the most commonly used P. pastoris vectors contain the very strong and tightly regulated alcohol oxidase (AOX1) promoter. The vectors also contain the *P. pastoris* histidinol dehydrogenase (HIS4) gene for selection in his4 hosts. Secretion of foreign protein require the presence of a signal sequence and the *S. cerevisiae* prepro alpha mating factor leader sequence has been widly and successfully used in Pichia expression system. Expression vectors are integrated into the P. pastoris genome to maximize the stability of expression strains. As in *S.cerevisiae*, cleavage of a *P.pastoris* expression vector within a sequence shared by the host genome (AOX1 or HIS4) stimulates homologous recombination events that efficiently target integration of the vector to that genomic locus. In general, a recombinant strain that contains multiple integrated copies of an expression cassette can yield more heterologous protein than single-copy strain. The most effective way to obtain high copy number transformants requires the transformation of Pichia recipient strain by the sphaeroplast technique (Cregg et all 1985, Mol.Cell.Biol. 5: 3376-3385) .

The preparation of the replicable expression vector may be carried out conventionally with appropriate enzymes for restriction, polymerisation and ligation of the DNA, by procedures described in, for example, Maniatis *et al.* cited above.

The recombinant host cell is prepared, in accordance with the invention, by transforming a host cell with a replicable expression vector of the invention under transforming conditions. Suitable transforming conditions are conventional and are described in, for example, Maniatis *et al.* cited above, or "DNA Cloning" Vol. II, D.M. Glover ed., IRL Press Ltd, 1985.

The choice of transforming conditions depends upon the choice of the host cell to be transformed. For example, in vivo transformation using a live viral vector as the transforming agent for the polynucleotides of the invention is described above. Bacterial transformation of a host such as *E. coli* may be done by direct uptake of the polynucleotides (which may be expression vectors containing the desired sequence) after the host has been treated with a solution of $CaCl_2$ (Cohen *et al.*, Proc. Nat. Acad. Sci., 1973, *69*, 2110) or with a solution comprising a mixture of rubidium chloride (RbC1), $MnCl_2$, potassium acetate and glycerol, and then with 3-[N-morpholino]-propane-sulphonic acid, RbC1 and glycerol. Transformation of lower eukaryotic organisms such as yeast cells in culture by direct uptake may be carried out by using the method of Hinnen et al (Proc. Natl. Acad. Sci. 1978, 75 : 1929-1933). Mammalian cells in culture may be transformed using the calcium phosphate co-precipitation of the vector DNA onto the cells (Graham & Van der Eb, Virology 1978, 52, 546). Other methods for introduction of polynucleotides into mammalian cells include dextran mediated transfection, polybrene mediated transfection, protoplast fusion, electroporation, encapsulation of the polynucleotide(s) into liposomes, and direct micro-injection of the polynucleotides into nuclei.

The invention also extends to a host cell transformed with a nucleic acid encoding the protein of the invention or a replicable expression vector of the invention.

Culturing the transformed host cell under conditions permitting expression of the DNA polymer is carried out conventionally, as described in, for example,

Maniatis *et al.* and "DNA Cloning" cited above. Thus, preferably the cell is supplied with nutrient and cultured at a temperature below 50°C, preferably between 25°C and 35°C, most preferably at 30°C. The incubation time may vary from a few minutes to a few hours, according to the proportion of the polypeptide in the bacterial cell, as assessed by SDS-PAGE or Western blot.

The product may be recovered by conventional methods according to the host cell and according to the localisation of the expression product (intracellular or secreted into the culture medium or into the cell periplasm). Thus, where the host cell is bacterial, such as *E. coli* it may, for example, be lysed physically, chemically or enzymatically and the protein product isolated from the resulting lysate. Where the host cell is mammalian, the product may generally be isolated from the nutrient medium or from cell free extracts. Where the host cell is a yeast such as *Saccharomyces cerevisiae* or *Pichia pastoris*, the product may generally be isolated from from lysed cells or from the culture medium, and then further purified using conventional techniques. The specificity of the expression system may be assessed by western blot using an antibody directed against the polypeptide of interest.

Conventional protein isolation techniques include selective precipitation, adsorption chromatography, and affinity chromatography including a monoclonal antibody affinity column. When the proteins of the present invention are expressed with a histidine tail (His tag), they can easily be purified by affinity chromatography using an ion metal affinity chromatography column (IMAC) column.

In a preferred embodiment of the invention the proteins of the present invention is provided with an affinity tag, such as a polyhistidine tail. In such cases the protein after the blocking step is preferably subjected to affinity chromatography. For those proteins with a polyhistidine tail, immobilised metal ion affinity chromatography (IMAC) may be performed. The metal ion, may be any suitable ion for example zinc, nickel, iron, magnesium or copper, but is preferably zinc or nickel. Preferably the IMAC buffer contains detergent, preferably a non-ionic detergent such as Tween 80, or a zwitterionic detergent such as Empigen BB, as this may result in lower levels of endotoxin in the final product.

Further chromatographic steps include for example a Q-Sepharose step that may be operated either before of after the IMAC column. Preferably the pH is in the range of 7.5 to 10, more preferably from 7.5 to 9.5, optimally between 8 and 9, ideally 8.5.

The amount of antigen in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each human dose will comprise 1-1000 g of antigen, preferably 1-500 g, preferably 1-100μg, most preferably 1 to 50μg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in vaccinated subjects. Following an initial vaccination, subjects may receive one or several booster immunisation adequately spaced. Such a vaccine formulation may be applied to a mucosal surface of a mammal in either a priming or boosting vaccination regime; or alternatively be administered systemically, for example *via* the transdermal, subcutaneous or intramuscular routes.

The amount of adjuvant such as Inv or ail protein adjuvant in the vaccine of the present invention is generally small, but depending on the vaccine formulation may be in the region of 1-1000 μg per dose, preferably 1-500 μg per dose, and more preferably between 1 to 100 μg per dose.

The composition of the present invention may be used for both prophylactic and therapeutic purposes. Accordingly, there is provided the use of a Yersinia adhesion protein in the manufacture of an adjuvant composition and in the manufacture of a vaccine for the prophylaxis and the treatment of viral, bacterial, parasitic infections, allergy, cancer and other non-chronic disorders. The present invention also provides for a method of treating a mammal susceptible to or suffering from an infectious disease or cancer, or allergy, or autoimmune disease. In a further aspect of the present invention there is provided a vaccine or adjuvant combination, comprising a Yersinia adhesion protein, preferably a Yersinia Inv or ail protein, as herein described for use as a medicament.

It is foreseen that compositions of the present invention will be used to formulate vaccines containing antigens derived from a wide variety of sources. For example, antigens may include human, bacterial, or viral nucleic acid, pathogen derived antigen or antigenic preparations, tumour derived antigen or antigenic preparations, host-derived antigens, including peptides derived from IgE, such as the histamine releasing decapeptide of IgE (known as the Stanworth decapeptide), recombinantly produced protein or peptides, and chimeric fusion proteins.

There is provided by the present invention a systemic vaccine composition comprising an antigen and a Yersinia adhesion protein adjuvant. Accordingly, there is provided a method of treatment of an individual susceptible to or suffering from a disease by the administration of a composition as substantially described herein through the systemic route of said individual. Also provided is a method to prevent an individual from contracting a disease selected from the group comprising infectious bacterial and viral diseases, parasitic diseases, prostate, breast, colorectal, lung, pancreatic, renal, ovarian or melanoma cancers; non-cancer chronic disorders, allergy, Alzheimer, atherosclerosis, comprising the administration of a composition as substantially described herein through the systemic route of said individual.

Alternatively, there is provided by the present invention a mucosal vaccine composition comprising an antigen and a Yersinia adhesion protein adjuvant. Accordingly, there is provided a method of treatment of an individual susceptible to or suffering from a disease by the administration of a composition as substantially herein described to a mucosal surface of said individual.

Furthermore, there is described a method of inducing a systemic antigen specific immune response in a mammal, comprising administering to a mucosal surface of said mammal a composition comprising an antigen and a Yersinia adhesion protein adjuvant. Further there is provided a method of manufacture of a vaccine, comprising taking a Yersinia adhesion protein adjuvant and admixing it with an antigen.

The invention will be further described, but not limited, by reference to the following examples:

EXAMPLE I:

Methods to formulate antigens and invasin

A method is described herein which allows the co-adsorption onto a suitable vehicle of recombinant invasin (*E. coli*-produced GST-invasin 397, supplied by Dr Autenrieth, Max von Pettenkofer Institute, Muënchen, Germany) and a model antigen, the lipided outer surface protein A (L-OspA) from *B. burgdorferi* (L-OspA was produced and purified as described in WO 99/61473). The GST-invasin 397 is obtainable by using the Glutathione S-transferase (GST) Gene Fusion System purchased from Amersham Pharmacia Biotech (catalog page 496). This system is an integrated system (pGEX) for the expression, purification and detection of GST-fusion proteins produced in *E. coli*. The invasin fragment is originating from *Yersinia enterocolitica*.

1. - Coating of proteins to latex beads

For noncovalent coating of beads, purified protein was dialyzed against PBS pH 7.0. About 10^9 latex beads (1 μm diameter, sulfate-modified; Molecular Probes or 1 μm diameter, reference LB11, Sigma) were washed with 1 ml of PBS and resuspended in 500 μl of PBS. Purified GST or GST-Inv397 fusion protein (0-2 mg) was added and allowed to adsorb to the beads for 3 h at room temperature (RT). After adding 500 μl of 20 mg/ml bovine serum albumin (BSA), the solution was incubated at room temperature for another 1 h. Then beads were washed in PBS containing 1 mg/ml BSA and stored at 4°C in 500 μl of PBS containing 0.2 mg/ml BSA. To determine the coupling efficiency, the protein concentration of the starting solution and of the supernatant before adding BSA was determined. Integrity of coated GST-Inv397 fusion protein was checked by Western Blot analysis.

2. - Co-adsorption of antigen and Yersinia Invasin onto Calcium phosphate

The following components are mixed under agitation for 1 hour at room temperature: $Ca_3(PO_4)_2$ supplied by Superfos Inc (2 mg within 200 μl NaCl 150 mM), L-OspA (100 μg within 109 μl NaCl 150 mM Phosphate10 mM, pH 6.8) and GST-invasin 397 (100 μg within 77 μl PBS $MgCl_2$ 2mM, pH 7). The formulation is then completed with 11 μl NaCl 1500 mM Phosphate 100 mM and 103 μl sterile water. The non-adsorbed material is controlled following centrifugation, using L-OspA-specific ELISA and bincinchoninic acid-based (micro BCA) protein dosage. According to these assays, 98% antigen and 50% invasin are adsorbed onto the vehicle, respectively.

3. – Comparison of adsorption of Invasin and Lipo-OspA on different particulate carriers

3.1. Adsorption of Y. invasin onto the carrier

The ratio protein / carrier for this adsorption assay was:

10 μg invasin / 100 μg carrier / 50 μl final volume

10 μg of invasin (GST-INV 397, 1300 μg/ml (Lowry), in PBS containing 2 mM $MgCl_2$, pH 7.0) were formulated with 100 μg carrier in a final volume of 50 μl. Near isoosmotic conditions were achieved by adjusting final formulations to 150 mM NaCl except for $FePO_4$ which is already produced in 128 mM PO_4 buffer. The sequence of adsorption was as follows (see Table 1): water for injection, 10x concentrated buffer, carrier, GST-INV. The mixture was stirred during 5 hours at room temperature, then overnight at 4°C, and subsequently centrifugated at 6000

rpm during 10 min. The supernatant was collected and a fraction of it (representing 0.5 μg invasin) assessed by SDS-PAGE (4-20% Tris-glycine Novex gel) in reducing conditions, followed by silver staining.

Table 1

	Based on cationic concentrations	Invasin	PO_4	NaCl	V total
$Al(OH)_3$	100 μg	10 μg	1.54 mM	150 mM	50 μl
$AlPO_4$	100 μg	10 μg	3.54 mM	150 mM	50 μl
$Ca_3(PO4)_2$	100 μg	10 μg	1.54 mM	150 mM	50 μl
$FePO_4$	+/- 100 μg	10 μg	128 mM		50 μl

Plain formulation ("plains") were also formulated as internal control. Plain formulations are constituted with the same ratio protein:carrier but only with carrier supernatant (no carrier), in order to prove there is no interference or adsorption with the buffer of the carrier.

3.2. Adsorption of Lipo-OspA onto the carrier

The ratio protein / carrier for this adsorption assay was:

100 μg LipoOspA / 1000 μg carrier / 500 μl final volume

Lipo-OspA protein has been adsorbed onto $Al(OH)_3$, $AlPO_4$, $Ca_3(PO_4)_2$, $Mg(OH)_2$, $FePO_4$. The corresponding "plain" (same formulation but without carrier) has been assessed in parallel.

100 μg of Lipo-OspA produced according to the method described in WO 99/61473 (OPA 140, 921 μg/ml (Lowry), in PO4 10 mM / NaCl 150 mM) were formulated with 1000 μg carrier in a final volume of 500 μl. Near isoosmotic conditions were

achieved by adjuvanting final formulations to 150 mM NaCl except for $FePO_4$ which is produced in 128 mM PO_4 buffer. The sequence of adsorption was as follows (see Table 2): water for injection, 10x concentrated buffer, carrier, Lipo-OspA. The mixture was stirred during 5 hours at room temperature, then overnight at 4°C, and subsequently centrifugated at 6000 rpm during 10 min. The supernatant was collected and a fraction of it (representing 0.5 μg Lipo-OspA) assessed by SDS-PAGE (12% Tris-glycine Novex gel) in reducing conditions, followed by silver staining.

Table 2

	Based on cationic concentrations	LipoOspA	PO_4	NaCl	V total
$Al(OH)_3$	1000 μg	100 μg	2.2 mM	150 mM	500 μl
$AlPO_4$	1000 μg	100 μg	4.2 mM	150 mM	500 μl
$Ca_3(PO4)_2$	1000 μg	100 μg	2.2 mM	150 mM	500 μl
$FePO_4$	+/- 1000 μg	100 μg	102.2 mM		500 μl

3.3. Co-adsorption of invasin and Lipo-OspA onto the carrier

The ratio protein / carrier for this adsorption assay was:

10 μg Invasin + 10 μg LipoOspA / 10 μg carrier / 50 μl final volume

10 μg of invasin + 10 μg of LipoOspA were formulated with 100 μg carrier in a final volume of 50 μl. In regard of previous results obtained in steps 1 and 2 described above, co-adsorption of those proteins with AlO(OH), $Ca_3(PO_4)_2$ and $FePO_4$ has been evaluated under three different sequences:

a) LipoOspA first on carrier, then invasin

b) Invasin first on carrier, then LipoOspA

c) Premix (lipoOspA + invasin) on carrier

Formulation.conditions are similar to those described above; to water were added (in the order of listing): 10X concentrated buffer, carrier, lipo-OpsA or invasin, stirring 5 hours at room temperature, incubation overnight at 4°C, addition of invasin or Lipo-OspA, stirring 5 hours at room temperature, incubation overnight at 4°C, centrifugation at 6000 rpm during 10 min). The supernatant was collected and a fraction of it (representing 0.5 µg Lipo-OspA + 0.5 µg invasin) assessed by SDS-PAGE (4-20% Tris-glycine Novex gel) in reducing conditions, followed by silver staining.

a) First LipoOspA, then invasin

			Lipo-Ospa	Invasin	NaCl 1500 mM	WFI	Total Vol.
$Al(OH)_3$	10380 µg / ml	9.63 µl	10.8 µl	7.7 µl	3.15 µl	18.72 µl	50 µl
$Ca_3(PO4)_2$	10000 µg / ml	10 µl	10.8 µl	7.7 µl	2.15 µl	19.35 µl	50 µl
$FePO_4$	+/-4422 µg/ml	31.5 µl	10.8 µl	7.7 µl	-	-	50 µl

b) First invasin, then LipoOspA

			Lipo-Ospa	Invasin	NaCl 1500 mM	WFI	Total Vol.
$Al(OH)_3$	10380 µg / ml	9.63 µl	10.8 µl	7.7 µl	3.15 µl	18.72 µl	50 µl
$Ca_3(PO4)_2$	10000 µg / ml	10 µl	10.8 µl	7.7 µl	2.15 µl	19.35 µl	50 µl
$FePO_4$	+/-4422 µg/ml	31.5	10.8 µl	7.7 µl	-	-	50 µl

		μl					

c) Premix (LipoOspA & Invasin), then co-adsorption on carrier

			Lipo-Ospa	Invasin	NaCl 1500 mM	WFI	Total Vol.
$Al(OH)_3$	10380 μg / ml	9.63 μl	10.8 μl	7.7 μl	3.15 μl	18.72 μl	50 μl
$Ca_3(PO4)_2$	10000 μg / ml	10 μl	10.8 μl	7.7 μl	2.15 μl	19.35 μl	50 μl
$FePO_4$	+/ 4422 μg/ml	31.5 μl	10.8 μl	7.7 μl	-	-	50 μl

3.4. Conclusion

As may be seen from Table 3, there is adsorption of Yersinia invasin (GST-INV) on most particulate carriers tested, $Al(OH)_3$, $Ca_3(PO_4)_2$, and $FePO_4$. No major adsorption was seen on $AlPO_4$. Lipo-OspA antigen was well adsorbed on all carriers. The integrity of invasin and of the Lipo-OspA antigen in the corresponding "plain" (same formulation but without carrier) formulation was well preserved in most formulations, although to a slightly lesser extent in $Al(OH)_3$.

As to the co-adsorption of Invasin and Lipo-OspA onto the carrier, we can conclude that:

1) On Al(OH)3 a good adsorption of both invasin and Lipo-OspA was achieved, which was not influenced by the sequence of addition of either components.

2) On $Ca_3(PO_4)_2$, a very good adsorption and a partial adsorption was achieved for Lipo-OspA and Invasin respectively, which was not affected by the sequence of addition of the components.

3) On $FePO_4$, the sequence of adsorption of either protein seems to be of critical importance, with the first component (whether Lipo-OspA or Invasin) being completely adsorbed, and the second component (whether invasin or Lipo-OspA) not adsorbed in the experimùental conditions assessed. When the premix (Lipo-OspA + invasin) was added to the carrier both were competing against each other for the adsorption sites onto the carrier, and as a result were partially adsorbed. When twice as much carrier was introduced into the reaction mixture, full adsorption of both proteins was achieved.

Table 3

Carriers	*LipoOspA*	*Invasin*
Al(OH)3	Good adsorption	Good adsorption
AlPO4	Good adsorption	No adsorption
Ca3(PO4)2	Good adsorption	Partial adsorption
FePO4	Good adsorption	Good adsorption

EXAMPLE II:

Techniques used to measure the antigen-specific antibody (Ab) responses

1. - ELISA for the measurement of OspA or tetanus toxoid (TT) specific serum IgG in mice

Maxisorp Nunc immunoplates are coated overnight at 4°C with 50 μl/well of 1 μg/ml L-OspA or TT diluted in PBS (in rows B to H of plate), or with 50 μl of 5 μg/ml purified goat anti-mouse Ig (Boerhinger), in PBS (row A). Free sites on the plates are blocked (1 hour, 37°C) using saturation buffer : PBS comtaining 1%BSA, 0.1% polyoxyethylene sorbitan monolaurate (TWEEN 20), and 4% Normal Bovine Serum (NBS). Then, serial 2-fold dilutions (in saturation buffer, 50 μl per well) of IgG isotype mixture added as a standard curve (mixture of mouse monoclonal antibodies IgG1, IgG2a and IgG2b from Sigma, starting at 200 ng/ml and put in row A) and serum samples (starting at a 1/100 dilution and put in rows B to H) are incubated for 1hr 30mins at 37°C. The plates are then washed (×3) with washing buffer (PBS, 0.1% polyoxyethylene sorbitan monolaurate (TWEEN 20)). Then, biotinylated goat anti-mouse IgG (Amersham) diluted 1/5000 in saturation buffer are incubated (50 μl/well) for 1hr 30 mins, at 37°C. After 3 washings, and subsequent addition of streptavidin-horseradish peroxidase conjugate (Amersham), plates are washed 5 times and incubated for 20 min at room temperature with 50 μl/well of revelation buffer (OPDA 0.4 mg/ml (Sigma) and H_2O_2 0.03% in 50mM pH 4.5 citrate buffer). Revelation is stopped by adding 50 μl/well H_2SO_4 2N. Optical densities are read at 492 and 630 nm by using Biorad 3550 immunoreader. Antibody titre are calculated by the 4 parameter mathematical method using SoftMaxPro software.

2. - ELISA for the measurement of OspA- and TT-specific IgA in fecal extracts, saliva and nasal washings of mice

Maxisorp Nunc immunoplates are coated overnight at 4°C with 50 μl/well of 1 μg/ml of L-OspA or TT, diluted in PBS (in rows B to H of plate), or with 50 μl of 5 μg/ml purified goat anti-mouse Ig (Boerhinger), in PBS (row A). Free sites on the plates are blocked (1 hour, 37°C) using saturation buffer : PBS containing 1%BSA, 0.1% polyoxyethylene sorbitan monolaurate (TWEEN 20), and 4% Normal Bovine

Serum (NBS). Then, serial 2-fold dilutions of IgA (monoclonal Ab MOPC315 from ICN Biomedicals), diluted in saturation buffer (50 μl per well) and added as a standard curve (starting at 200 ng/ml and put in row A), and fecal, saliva or nasal washings extracts (starting at a 1/2 dilution and put in rows B to H) are incubated for 1hr 30 mins at 37°C. The plates are then washed (×3) with washing buffer (PBS, 0.1% polyoxyethylene sorbitan monolaurate (TWEEN 20)). Then, biotinylated goat anti-mouse IgA (Amersham) diluted 1/5000 in saturation buffer are incubated (50 μl/well) for 1hr 30mins, at 37°C. After 3 washings, and subsequent addition of streptavidin-horseradish peroxidase conjugate (Amersham), plates are washed 5 times and incubated for 10 min at room temperature with 50 μl/well of revelation buffer (TMB single component from Biorad). Revelation is stopped by adding 50 μl/well H_2SO_4 0.4N. Optical densities are read at 450 and 630 nm by using Biorad 3550 immunoreader. Antibody titre are calculated by the 4 parameter mathematical method using SoftMaxPro software.

A similar technique is used to measure total IgA in fecal, saliva or nasal washings extracts. In this, goat anti-mouse Ig polyclonal Abs are coated on the entire plate. Revelation is done with OPDA 0.4 mg/ml (Sigma) and H_2O_2 0.03% in 50mM pH 4.5 citrate buffer. Optical densities are read at 492 and 630 nm.

IgA responses are expressed as μg antigen-specific IgA / mg total IgA.

3. - Inhibition assay for the measurement of serum LA2-like antibody titres to lipo-OspA

Antibody titres in the vaccinees were studied with respect to their LA2-like specificity. LA2 is a murine monoclonal antibody which recognizes a conformational OspA epitope at the surface of the bacteria and has been shown to be able to kill B. burgdorferi in vitro, as well as to protect mice against a challenge with laboratory-grown spirochete (Schaible UE et al. 1990. Proc Natl Acad Sci USA 87:3768-3772). Moreover, LA-2 mab has been shown to correlate with bactericidal antibodies, and studies on human sera showed also a good correlation between the total anti-OspA IgG titers and the LA-2 titers (as measured by ELISA).

Maxisorp Nunc immunoplates are coated overnight at 4°C with 50 μl/well of 0.5μg/ml lipo OspA diluted in PBS. Free sites were blocked with saturation buffer for 1hr at 37°C with (100 μl/well of saturation buffer: PBS/ BSA 1%/ Tween 20 0.1%/ NBS 4%). Serial 2-fold dilutions of LA2 monoclonal Ab (mAb) starting at 4 μg/ml were diluted in saturation buffer (50 μl per well) to form a standard curve. Dilutions of serum samples from the vaccinees (starting at a 1/10 dilution) were also added and the plates incubated for 2hrs at 37°C. The plates were washed after incubation 3 times with PBS/ TWEEN 20 (0.1%). LA2 mAb-peroxidase conjugate (1/10,000) diluted in saturation buffer was added to each well (50 μl/well) and incubated for 1hr at 37°C. After 5 washings, plates are incubated for 20 min at room temperature (in darkness) with 50 μl/well of revelation buffer (OPDA 0.4 mg/ml and H_2O_2 0.03% in 50mM pH 4.5 citrate buffer). The reaction and colour formation was stopped with H_2SO_4 2N. Optical densities are read at 492 and 630 nm by using Biorad 3550 immunoreader. LA2-like Ab titers are calculated by the 4 parameter mathematical method using SoftMaxPro software. LA2-like antibody titres were determined by comparison with the standard curve.

EXAMPLE III:

Animal models to assess the immune response

1. Intraduodenal immunization in mice

Female Balb/c mice aged 8 weeks (n = 10) are immunised intraduodenally (by direct injection inside the duodenum after small incision of the skin and the peritoneal membrane) at days 0, 14 and 28 with 10 μg L-OspA contained in 50 μl of A: PBS; B: GST-invasin 397 10 μg; or, co-adsorbed with 10 μg GST-invasin 397 onto C: 100 μg Al(OH)3; D: 200 μg $Ca_3(PO_4)_2$; E: 200 μg $FePO_4$, as described in the Example 1 (under second paragraph). Antibody responses are measured in sera and fecal extracts (see the Example 2) after each immunization.

The same formulations as above, but containing TT as an antigen, are tested in a second experiment.

2. Buccal immunization in mice

Female Balb/c mice aged 8 weeks (n = 10) are immunised buccally at days 0, 14 and 28 with 10 μg L-OspA contained in 50 μl of A: PBS; B: GST-invasin 397 10 μg; or, co-adsorbed with 10 μg GST-invasin 397 onto C: 100 μg Al(OH)3; D: 200 μg $Ca_3(PO_4)_2$; E: 200 μg $FePO_4$,as described in the Example 1 (under second paragraph). Antibody responses are measured in sera, saliva and fecal extracts (see the Example 2) after each immunization.

In a second experiment, the same formulations as above are administered as freezed-dried powders containing or not an antacid (calcium carbonate).

The same formulations as in experiments 1 and 2, but containing TT as an antigen, are tested in a third (as liquid formulations) and a fourth (as freezed-dried formulations) experiment, respectively.

3. Buccal immunization in rabbits

To take advantage of the presence of palatine tonsils in rabbits, similar experiments as those described in the previous paragraph are done in this species. ELISA assays are basically the same as those used in mice, except that secondary antibody reagents are specific of rabbit immunoglobulins.

4. Intranasal immunization in mice

Female Balb/c mice aged 8 weeks (n = 10) are primed intramuscularly at day 0 with 1 μg L-OspA adsorbed onto 50 μg alum. They are intranasally boosted (under anesthesia) at day 28 with 2 μg L-OspA contained in 10 μl of A: PBS; B: GST-invasin 397 2 μg; or, co-adsorbed with 2 μg GST-invasin 397 onto C: 20 μg Al(OH)3; D: 40 μg $Ca_3(PO_4)_2$; E: 40 μg $FePO_4$,as described in the Example 1 (under second paragraph). Antibody responses are measured in sera and nasal washings (see the Example 2) after each immunization.

The same formulations as above, but containing TT as an antigen, are tested in a second experiment.

CLAIMS

1. An adjuvant composition comprising an adhesion protein from the Yersinia genus or a fragment thereof.
2. An adjuvant composition according to claim 1, wherein the adhesion protein is encoded by the inv gene of *Yersinia pseudotuberculosis*, the inv gene of *Yersinia enterocolitica* or by the ail gene of *Yersinia enterocolitica*.
3. An adjuvant composition according to claims 1 and 2 optionally comprising a carrier.
4. An adjuvant composition according to claim 4 wherein the carrier is selected from the group comprising: a metallic salt particle such as aluminium phosphate, aluminium hydroxyde, calcium phosphate, magnesium phosphate, iron phosphate, calcium carbonate, magnesium carbonate, calcium sufate, magnesium hydroxyde, or double salts like ammonium-iron phosphate, potassium-iron phosphate, calcium-iron phosphate, calcium-magnesium carbonate, or a mix of any of those salts; or a porous polymeric particle, such as a microbead or a nanoparticle.
5. An adjuvant composition according to any of claims 1 to 4 additionally comprising another immunostimulant selected from the group comprising 3D-MPL, QS21, CpG or a polyoxyethylene ether or ester.
6. A vaccine composition comprising an adjuvant as claimed in any of claims 1 to 5 further comprising an antigen or an antigen composition.
7. A vaccine composition according to claim 6 wherein the antigen is linked to the adjuvant of any of claims 1 to 5 through direct or indirect linkage.
8. A vaccine composition according to claim 6 wherein the antigen is not linked to the adjuvant of any of claims 1 to 5.
9. A vaccine composition as claimed in any of claims 6 to 8, wherein said antigen or antigenic composition is selected from the group comprising: Human Immunodeficiency Virus, Varicella Zoster virus, Herpes Simplex Virus type 1, Herpes Simplex virus type 2, Human cytomegalovirus, Dengue virus, Hepatitis A, B, C or E, Respiratory Syncytial virus, human papilloma virus, Influenza virus, Hib, Meningitis virus, Salmonella, Neisseria, Borrelia, Chlamydia,

Bordetella, Enterotoxic E. coli, Campylobacter, Streptococcus, Moraxella, Mycoplasma, Mycobacteria, Haemophilus, Plasmodium or Toxoplasma, Stanworth decapeptide; or Tumour associated antigens (TAA), MAGE, BAGE, GAGE, MUC-1, Her-2 neu, LnRH, CEA, PSA, PSMA, PAP, prostase, KSA, tyrosinase or PRAME.

10. A vaccine as claimed in claim 9, wherein said antigen is selected from the group comprising Lipo-OspA from Borrelia burgdorferi, Campylobacter whole cells or tetanus toxoid.

11. A vaccine composition according to any of claims 6 to 10 wherein the adjuvant:antigen ratio is from about 1:1000 to about 1000:1.

12. A vaccine composition according to any of claims 6 to 11 further comprising a pharmaceutically acceptable excipient or diluent.

13. The use of a Yersinia adhesion protein in the manufacture of an adjuvant composition.

14. The use of a Yersinia adhesion protein in the manufacture of a mucosal vaccine for the treatment of viral, bacterial, parasitic infections, allergy, or cancer.

15. A vaccine or adjuvant as claimed herein for use as a medicament.

16. A process for making a vaccine composition according to any of claims 6 to 12, comprising admixing a Yersinia adhesion protein and an antigen or antigenic composition.

17. A method of inducing a systemic antigen specific immune response in a mammal, comprising administering to a mucosal surface of said mammal a composition comprising an antigen and an adjuvant according to any of the claims 1 to 5.

18. A method of treating a mammal suffering from or susceptible to a pathogenic infection, or cancer, or allergy, comprising the administration of a non-toxic and effective amount of a vaccine composition as claimed in any of claims 6 to 12.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 01/03786

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/39 A61P31/00 A61P33/00 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, CHEM ABS Data, WPI Data, MEDLINE, CANCERLIT, EMBASE, LIFESCIENCES

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00 18354 A (OAKS EDWIN V ;TURBYFILL KEVIN ROSS (US); WALTER REED ARMY INST OF) 6 April 2000 (2000-04-06)	1,3,6,8, 12-18
Y	abstract page 1, line 8 - line 16 page 3, line 20 -page 4, line 33 page 6, line 3 - line 18 page 9, line 5 - line 10 page 14, line 21 - line 32 page 29, line 9 -page 30, line 9 page 36, line 4 - line 26 claims 1,2 ---	2
Y	US 5 338 842 A (FALKOW STANLEY ET AL) 16 August 1994 (1994-08-16) cited in the application the whole document ---	2
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

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Date of the actual completion of the international search	Date of mailing of the international search report
25 March 2002	02/04/2002

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 239 066 A (FALKOW STANLEY ET AL) 24 August 1993 (1993-08-24) the whole document	2
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